Research Article

Soluble Expression of Recombinant Nerve Growth Factor in Cytoplasm of *Escherichia coli*

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Background: Pivotal roles of Nerve growth factor (NGF) in the development and survival of both neuronal and non-neuronal cells indicate its potential for the treatment of neurodegenerative diseases. However, investigation of NGF deficits in different diseases requires the availability of properly folded human β-NGF. In previous studies bacterial expression of hNGF demonstrated the feasibility of its overproduction. However, known limitations in the use of *E. coli* as an expression host for a protein with three intra-chain disulfide bonds were evident.
Objectives: Here an optimized system was developed to overexpress the soluble NGF in *E. coli*.
Materials and Methods: The gene encoding the β subunit of mature hNGF was optimized based on *E. coli* codon preference and cloned into pET-32a expression vector providing His- and Trx- tags for detection and increasing the solubility of recombinant protein, respectively. The recombinant DNA was expressed in *E. coli* Origami (DE3), which enhances the correct formation of disulfide bonds in the cytoplasm of *E. coli*. Different culture conditions were evaluated to increase soluble expression of the target protein.
Results: The highest soluble expression level was achieved when *E. coli* Origami (DE3) cells expressing NGF were grown at 30°C in TB medium with 0.2 mM IPTG induction at OD_{600nm} = 1 for 4 h.
Conclusions: Our results indicated that the recombinant NGF was successfully expressed as a soluble form.

Keywords: Codon Preference Optimization; *E. coli*; Nerve Growth Factor; Recombinant Protein production; Trx-tag Fusion

1. Background

Neurotrophins are a family of proteins which play vital roles in the development and maintenance of neurons in peripheral and central nervous systems. This family includes Nerve Growth Factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (1, 2).

NGF is the best-described member of neurotrophin family and was originally identified by Hamburger and Levi-Montalcini in the 1950s (3). NGF supports growth, differentiation and maintenance of peripheral and central nervous systems (1, 3). The complete NGF molecule (140 kDa) is composed of α, β and γ subunits. However, neuronotrophic activity relates exclusively to β subunit, a homodimer protein with a molecular weight of approx. 26.5 kDa. Transcription and subsequently translation of the β-*NGF* results in the formation of a 241 amino-acids prepro-protein. The pre-peptide consisting of 18 amino acids is cleaved upon translocation into the endoplasmic reticulum. The pro-peptide composing of 103 residues is subsequently removed by cellular convertases (4). X-ray crystallography structure of NGF revealed that the NGF monomer is an elongated molecule consisted of three hairpin loops, a cysteine-knot motif and two pairs of twisted, antiparallel β-strands. The cysteine-knot motif stabilizes the NGF structure. In the biologically active form, two monomers are arranged in a parallel manner to form a homodimer (5).

The proposed investigation and treatment of NGF deficits in different neurodegenerative diseases such as Alzheimer require the availability of properly folded human β-NGF. Isolation of proteins from natural

sources is difficult and don't meet the requirements for quantity. Recombinant technology using different expression hosts is the method of choice. Among all recombinant expression hosts, *E. coli* is the most conventional organism for the recombinant production of useful proteins (6). Its advantages include extensive available knowledge of the genetics of the bacterium, low cost of growth medium, fast cell growth, high levels of target gene expression, ease of use and scalability of experiments as well as the great number of plasmids and vectors developed for this system (7). Therefore not surprising that *E. coli* system is most commonly used for industrial and pharmaceutical protein production. However, the use of *E. coli* in this field has several drawbacks. For example, many of the eukaryotic post-translational modifications are absent in *E. coli* (8). Therefore, heterologous gene expression in *E. coli* results in the production of insoluble proteins mainly called inclusion bodies, which require further efforts to be refolded (9).

In reported studies bacterial expression of human NGF demonstrated the feasibility of overproduction but also limitations in the use of *E. coli* as an expression host for a protein with three intra-chain disulfide bonds (10-16). Isolation and purification of hNGF from inclusion bodies require solubilization followed by refolding. However, because of the three intrachain disulfide bonds of hNGF, which their proper formation is essential for biological activity, this process can be inefficient and difficult. The reduced environment of *E. coli* cytoplasm is inappropriate for the formation of disulfide bonds. This environment is the result of glutathione reductase (gor) and thioredoxin reductase (trxB) actions in cytoplasm (17).

Disulfide bonds mainly form in the periplasm of *E. coli*, which is a more oxidizing environment due to the presence of Dsb proteins including DsbA, B, C, D and G (18). The cytoplasm of *E. coli* can be genetically engineered to provide a suitable situation for successful expression of proteins containing disulfide bonds. Correct cytoplasmic expression of proteins results in significantly higher protein yields relative to periplasmic expression of disulfide-rich proteins (19).

During the past decades considerable progress and development in genetic engineering and manipulation have improved the soluble and functional production of recombinant proteins in *E. coli* (20).

To the best of our knowledge, hNGF expression in *E. coli* cytoplasm has led to aggregated proteins (10-16, 21). Thus, the soluble expression of hNGF in *E. coli* has been considered a challenging task.

2. Objectives

The objective of this study was to develop an optimized system to overexpress the soluble NGF in *E. coli* cytoplasm and avoid the problems associated with inclusion body denaturation and refolding. The mature human β-NGF coding sequence was optimized based on *E. coli* codon preference and cloned into pET-32a expression vector providing His- and Trx- tags for detection and increasing the solubility of recombinant protein, respectively. The recombinant DNA was expressed in *E. coli* Origami (DE3). Different culture conditions were evaluated to increase the soluble expression of NGF (8).

3. Materials and Methods

3.1. Plasmid and Bacterial Strains

pET-32a plasmid (Novagen Inc., Madison, Wis.) was selected to provide Trx-tag (solubility tag) and His-tag (to help detection and purification stages) at the N-terminus of the target gene. *E. coli* strains DH5α and Origami (DE3) were the products of Novagen (Novagen Inc., Madison, WI).

3.2. Construction of Expression Plasmid

Mature human *NGF* was optimized based on the *E. coli* codon usage (called *meNGF* thereafter, Figure 1) and synthesized by MWG-Biotech (Ebersberg, Germany). *Nco*I and *Hind*III sites were designed at the 5′ and 3′-ends, respectively. The synthesized target gene was inserted in to *Hind*III/*Nco*I sites of pET-32a plasmid. Recombinant plasmid pET32a-meNGF was transformed into the competent *E. coli* DH5α cells. The obtained construct was verified by restriction endonuclease digestion and DNA sequencing.

3.3. MeNGF Expression and Optimization

To express the recombinant β-NGF, pET32a-meNGF plasmid was transformed into competent *E. coli* Origami (DE3). Transformed cells were grown at 37ºC in LB containing 100 $\mu g.mL^{-1}$ Ampicillin, 15 $\mu g.mL^{-1}$ kanamycin, 50 $\mu g.mL^{-1}$ Streptomycin and 12.5 $\mu g.mL^{-1}$ Tetracycline until OD_{600nm}= 1, followed by induction with 0.2 mM and 1 mM IPTG (Fermentas, Burlington, Canada). The cells were harvested after 4 h and analyzed for protein expression by SDS-PAGE.

To reach the highest possible level of soluble meNGF expression, several experimental parameters affecting the rate of protein expression including culture media (LB, TB and 2X YT), the effect of glucose,

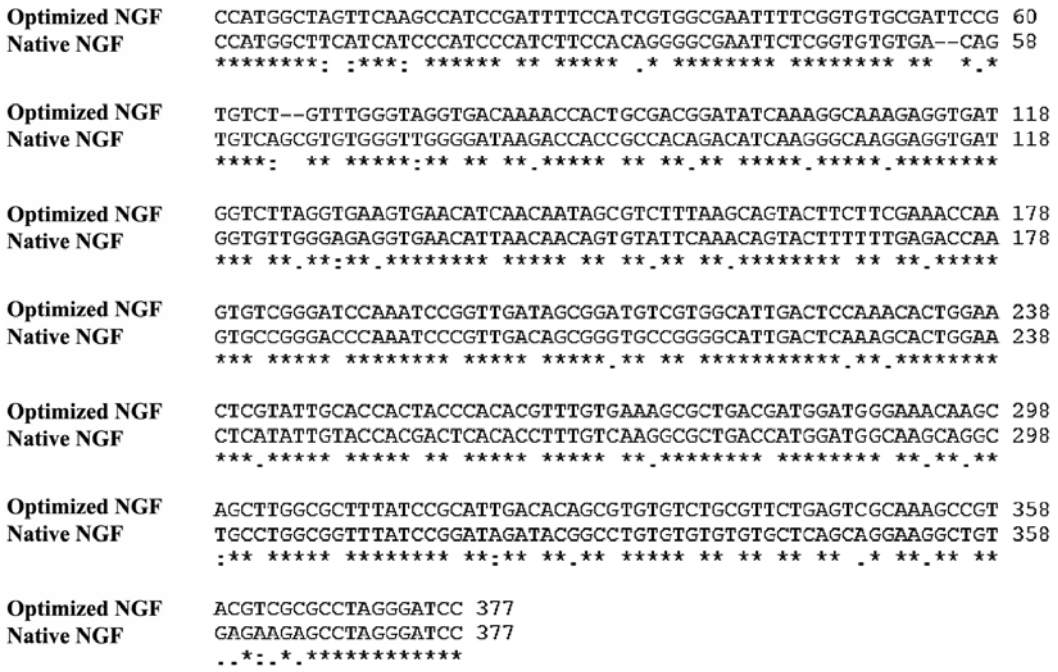

Figure 1. Alignment of nucleotide sequences of native and optimized mhNGF

induction stage (OD_{600nm}= 0.5, 1 and >1), and induction temperature (24, 30 and 37ºC) were analyzed for single clone by one factor at a time method. To extract the expressed meNGF protein, cells were harvested and kept at -20ºC for more than 30 min. For protein extraction under native conditions, the cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole) and incubated on ice for 15-30 min. Cell suspension was sonicated (10 short burst of 10 s followed by intervals of 30 s of cooling) and centrifuged (12000 ×g, 20 min, 4ºC). Supernatant was collected for SDS-PAGE analysis of soluble portion.The amount of soluble meNGF in different culture conditions was compared based on the intensity of related band on SDS-PAGE gel and quantitative analysis with Alpha Ease FC software.

3.4. Western Blot Analysis

Proteins were separated by 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked in Skim milk 5%/ TBS-T (Tris-Buffered Saline containing 0.5% v/v Tween 20) for 16 h at 4ºC and washed three times with TBS-T. The nitrocellulose membrane was incubated for 2 h at room temperature with conjugated anti His-tag antibody diluted 1:2000 in TBS-T. The membrane was washed with TBS-T three times and treated using H_2O_2 and DAB solution (10 mM $NiCl_2$ was also added) (22) and placed in darkness until the appearance of the protein band (23).

3.5. Partial Purification of Recombinant Protein

E. coli Origami (DE3) containing pET32a-meNGF plasmid was grown in large scale in the best culture condition (grown at 37ºC in TB containing appropriate antibiotics until OD_{600nm}= 1, followed by induction with 0.2 mM IPTG and incubation at 30ºC). The pellet of bacterial cells expressing meNGF was harvested and resuspended in lysis buffer. Cell suspension was sonicated and centrifuged at 12000 ×g, 30 min, and 4ºC. The supernatant was collected and recombinant meNGF protein was purified from supernatant under native conditions via His-tag using TALON cobalt metal affinity chromatography according to the manufacture's instruction (Clontech Laboratories, Inc.USA). The output fractions were analyzed by SDS-PAGE following Western analysis.

4. Results

4.1. Identification of Recombinant PET32a-meNGF Plasmid

The integrity of the recombinant pET32a-meNGF was confirmed by double digestion using *Hind*III and

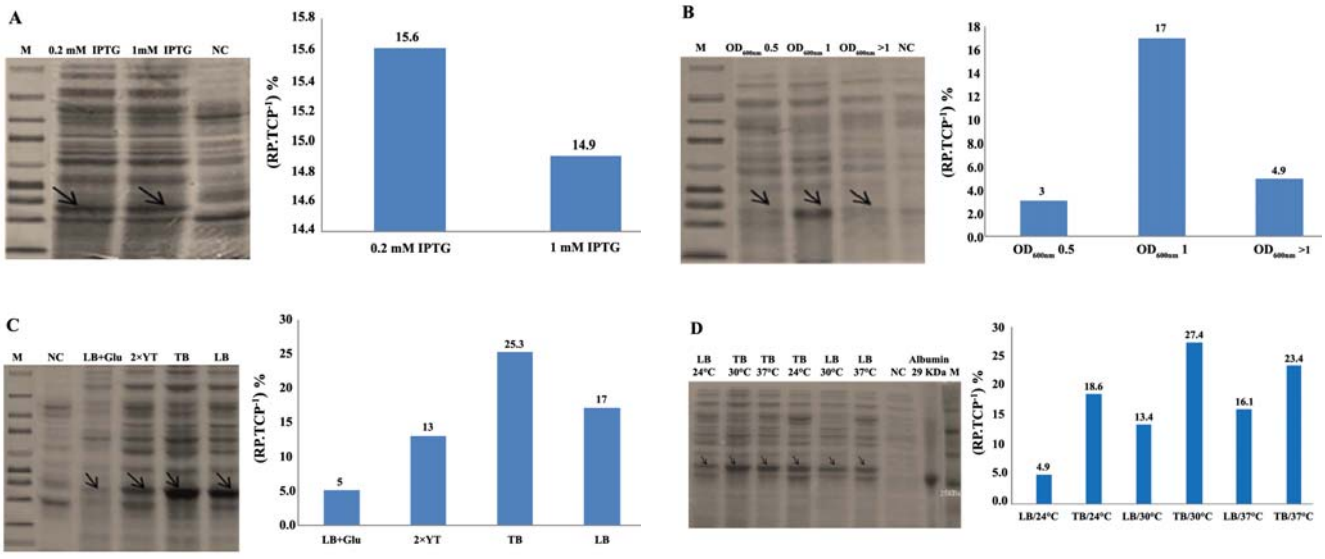

Figure 2. SDS-PAGE analysis of recombinant meNGF expression with *Coomassie blue*-stained. In all conditions Negative Control (NC) is *E. coli* Origami (DE3) lacking recombinant plasmid. M: Protein marker (From up to down: 260/140/100/70/50/40/35/25/15/10kDa.) Recombinant protein (is shown with arrow). A: Effect of IPTG concentration on expression level of meNGF. B: Effect of induction time on expression level of meNGF. C: Media optimization for high expression level of meNGF. D: Effect of media and incubation temperature on expression level of meNGF. RP/TCP: Recombinant protein/Total cell protein

*Nco*I restriction enzymes. Identity and orientation of meNGF in the construct were confirmed by sequencing the recombinant plasmid.

4.2. meNGF Expression

The T7 promoter of recombinant pET32a-meNGF plasmid in *E. coli* Origami (DE3) single clone grown at 37ºC in LB was induced by IPTG at different concentration (Figure 2A). Based on the obtained results, 0.2 mM IPTG concentration was selected for further analysis. OD_{600nm} value is a parameter indicating the physiological condition of *E. coli* cells. Amongst tested bacterial optical densities (0.5, 1 and >1), the most suitable induction time was OD_{600nm}= 1 (Figure 2B).

To optimize media for high expression level of meNGF, LB, LB containing glucose (6 $g.L^{-1}$), 2×YT and TB media were tested. Cells grown for 4 h at 37°C in these media containing appropriate antibiotics until OD_{600nm} reached 1, followed by IPTG induction at 0.2 mM concentration. As it's shown in Figure 2C, TB and LB media were more appropriate for meNGF protein expression. Different incubation temperatures (24, 30,

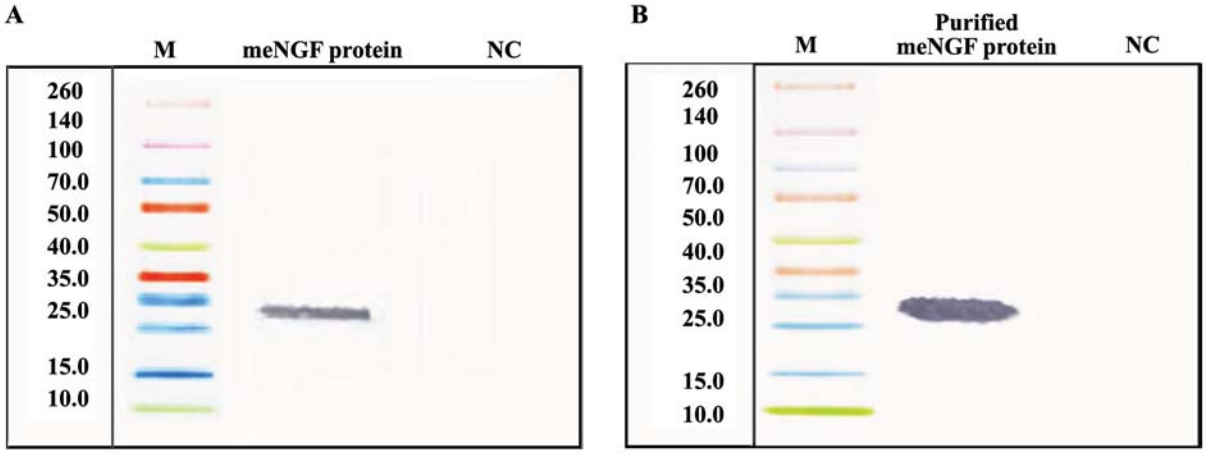

Figure 3. Western blot analysis of recombinant meNGF protein (with HRP anti-His tag antibody). A: before purification. B: after purification. M: Protein marker. NC: Negative control

and 37°C) along with media effect (LB and TB media) on the level of protein expression were investigated under the optimum parameters obtained from our experiments. The results show that at all incubation temperatures the expression level seems to be higher in TB compared to LB (Figure 2D). In all conditions, negative control was *E. coli* Origami (DE3) lacking recombinant plasmid.

4.3. Western Blot Analysis

Western analysis was carried out to confirm the expression of meNGF protein. Since the expression level of meNGF in TB medium at 30°C was higher than the others, it was selected for western blot analysis (Figure 3A).

4.4. Partial Purification of Recombinant Protein

meNGF was expressed better at 30°C in TB at OD_{600nm}=1, followed by induction with 0.2 mM IPTG. Recombinant protein purification was performed under native condition (Figure 3B).

5. Discussion

NGF, the first discovered and the best-known member of the neurotrophin family, has pivotal roles in the development, survival and proliferation of both neuronal and non-neuronal cells (24-26). Further investigation of NGF requires the availability of properly folded human β-NGF. In previous studies bacterial expression of recombinant human NGF demonstrated the feasibility of overproduction but also limitations in the use of *E. coli* as a host for expression of a protein with three intra-chain disulfide bonds. Although the other protein expression systems such as yeast, insect and mammalian cells usually produce functional protein; however, production is cost- and time-consuming and the yield is low (27). In addition, *in vivo* systems are not appropriate for the expression of toxic proteins.

The specific aim of this study was directed towards obtaining significant amounts of recombinant human NGF (rhNGF) in soluble state in one step from *E. coli* cells and avoiding problems associated with inclusion body denaturation and refolding.

Codon bias is one the biggest obstacles for recombinant protein expression in *E. coli*. The differences between the codon usage of the target gene and expression host lead to amino acid misincorporation and truncation of the polypeptide chain (28, 29). Selection of the vector is another most important issue affecting essential variables for the success of gene expression, including (1) target protein localization in *E. coli* cell (2) copy number of plasmid (3) type of promoter (4) fused tags, which influence protein solubility and stability (8). The failure of NGF production as a soluble and functional protein in *E. coli* is due to the absence of post-translational modifications required for disulfide-bond formation. Disulfide-bond formation occurs in the periplasm of *E. coli*, which is a more oxidizing compartment due to the presence of Dsb proteins including Dsb A, B, C, D and G (18). However, the cytoplasm of *E. coli* can be genetically engineered to provide a suitable situation for successful expression of proteins containing disulfide bonds. In compare to periplasmic expression level, protein yields will be significantly higher when correct folding occurs in cytoplasm (19). As mentioned previously, the reducing environment of the cytoplasm is maintained by Trx and GSH systems; therefore, the mutation of these pathways leads to an oxidizing cytoplasm. This rationale has been exploited by Novagen, who have developed and commercialized single (trxB) and double (trxB–/gor–) mutants of *E. coli* as AD494 and OrigamiTM host strains (OrigamiTM, OrigamiTM B, Rosetta-gamiTM, Rosetta-gamiTM 2), respectively. These mutants enhance the correct formation of disulfide bonds in the cytoplasm of *E. coli*.

Another common strategy to express the target protein in a soluble state is to evaluate different culture conditions, such as inducer concentration, time of induction, cell growth conditions, media composition (8). Reducing the synthesis rate of the target gene product by decreasing the concentration of inducer is a traditional approach to promote proper folding (30). OD_{600nm} value is a parameter indicating the physiological condition of *E. coli* cells. *E. coli* cells can grow on different media such as LB, 2YT, terrific broth (TB), and minimal media (M9). TB medium (Terrific Broth) is a phosphate buffered rich medium. In addition to 20% more peptone and 38% more yeast extract than in LB, TB also has 0.4% glycerol which is an extra carbon source (a carbohydrate source that unlike glucose is not fermented to acetic acid) and also acts as a chemical chaperone to improve the proper folding of proteins in *E. coli* cytoplasm (Expression Technologies Inc.). Temperature is another effective parameter in recombinant protein production. Optimal growth temperature for *E. coli* is 37ºC. *E. coli* cells cannot grow well at temperatures higher than 42ºC and lower temperatures reduce their growth rate. Soluble proteins are often produce at temperatures from 15 to 30°C (31), since protein synthesis slows at lower temperatures and inclusion body accumulation is

decreased (30).

To avoid codon bias obstacle the gene encoding mature human β-NGF was optimized based on *E. coli* codon usage and called meNGF thereafter. The synthesized meNGF was inserted into pET-32a plasmid which provides Trx-tag to increase the solubility and stability of recombinant protein and also His-tag to help detection and purification of expressed recombinant protein. To overcome protein disaggregation problem associated with three disulfide bridges of NGF monomer, *E. coli* Origami (DE3) was chosen as the expression host and our recombinant construction was transformed to competent cells. Evaluation of different culture conditions indicated that the highest soluble expression level was achieved when *E. coli* Origami (DE3) cells expressing meNGF were grown at 30°C in TB medium with 0.2 mM IPTG induction at OD_{600nm}= 1 for 4 h.

Overall, the parameters considered in this study and lead to obtaining high and acceptable amounts of soluble meNGF are gene optimization based on *E. coli* codon usage; selection of a suitable vector to enhance soluble expression; selection of a more appropriate host for expressing disulfide-bond containing proteins in *E. coli* cytoplasm; optimization of culture condition parameters including IPTG concentration, induction stage, culture media, and incubation temperature after induction.

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